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Integrated multi-biomarker responses of juvenile seabass to diclofenac, warming and acidification co-exposure

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ABSTRACT

Pharmaceutical drugs, such as diclofenac (DCF), are frequently detected in the marine environment, and recent evidence has pointed out their toxicity to non-target marine biota. Concomitantly, altered environmental conditions associated with climate change (e.g. warming and acidification) can also affect the physiology of marine organisms. Yet, the underlying interactions between these environmental stressors (pharmaceutical exposure and climate change-related stressors) still require a deeper understanding. Comprehending the influence of abiotic variables on chemical contaminants' toxicological attributes provides a broader view of the ecological consequences of climate change. Hence, the aim of this study was to assess the ecotoxicological responses of juvenile seabass *Dicenthrachus labrax* under the co-exposure to DCF (from dietary sources, 500 ± 36 ng kg^{-1} dw), warming ($\Delta T^{o}C = +5\,^{\circ}C$) and acidification ($\Delta pCO_2 \sim 1000$ µatm, equivalent to $\Delta pH = -0.4$ units), using an "Integrated Biomarker Response" (IBR) approach. Fish were exposed to these three stressors, acting alone or combined, for 28 days in a full cross-factorial design, and blood, brain, liver and muscle tissues were subsequently collected in order to evaluate: i) animal/organ fitness; ii) hematological parameters and iii) molecular biomarkers. Results not only confirmed the toxicological attributes of dietary exposure to DCF in marine fish species at the tissue (e.g. lower HSI), cellular (e.g. increased ENAs and lower erythrocytes viability) and molecular levels (e.g. increased oxidative stress, protein degradation, AChE activity and VTG synthesis), but also showed that such attributes are altered by warming and acidification. Hence, while acidification and/or warming enhanced some effects of DCF exposure (e.g. by further lowering erythrocyte viability, and increasing brain GST activity and Ub synthesis in muscle), the co-exposure to these abiotic stressors also resulted in a reversion/inhibition of some molecular responses (e.g. lower CAT and SOD inhibition and VTG synthesis). IBRs evidenced that an overall higher degree of stress (i.e. high IBR index) was associated with DCF and warming co-exposure, while the effects of acidification were less evident. The distinct responses observed when DCF acted alone or the animals were co-exposed to the drug together with warming and acidification not only highlighted the relevance of considering the interactions between multiple environmental stressors in ecotoxicological studies, but also suggested that the toxicity of pharmaceuticals can be aggravated by climate change-related stressors (particularly warming), thus, posing additional biological challenges to marine fish populations.

1. Introduction

Over the last decades, the consumption of pharmaceutically active compounds (PhACs) has increased drastically, reaching an average worldwide consumption of over 100,000 tons per year, and an average *per capita* consumption of 150 g per year in developed countries

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(Lonappan et al., 2016). As the removal of PhACs by waste water treatment plants (WWTPs) is still limited, many compounds and their metabolites are continuously introduced in the aquatic environment (concentrations ranging from ng L^{-1} up to low mg L^{-1}), potentially representing a risk to non-target marine species (e.g. Gros et al., 2012; Gaw et al., 2014; Mezzelani et al., 2016).

Widely known as one of the most popular "pain-killers", diclofenac (DCF; usually available in the forms of sodium or potassium salts) is a non-steroidal anti-inflammatory drug (NSAID) commonly prescribed to reduce inflammation and/or to relieve pain induced by different chronic diseases (e.g. arthritis) or injuries. Despite its intensive usage, in both humans (being in the top 50 list of most prescribed and sold pharmaceuticals; European Medicines Agency, 2013; ARSLVT, 2015) and stockbreeding (i.e. bovine and pork farming; European Medicines Agency, 2014), along with its inefficient removal at WWTPs (its parental form is removed by between 30 and 70%, leaving levels in wastewater samples in the order of µg L^{-1}; Lonappan et al., 2016), DCF's presence in aquatic systems remains unregulated in the European Union. Still, the European Commission has recently placed DCF under the "Watch List" of emerging non-regulated aquatic pollutants, for which further monitoring and toxicological data are needed, to accurately estimate their ecological risks and decide, whether their presence in the environment (and seafood) should in the future be regulated or not (proposed maximum allowable DCF concentration of 0.1 µg/L and 0.01 µg/L in freshwater and seawater, respectively; Decision EU, 2015/495).

Although several studies have recently pointed out DCF's toxicity to marine species (Gonzalez-Rey and Bebianno, 2014; Mezzelani et al., 2016; Gröner et al., 2017), the ecotoxicological implications of this compound are still far from being completely clarified, largely for two reasons. First, most studies carried out so far were focused on DCF exposure via water (e.g. Munari et al., 2016; Boisseaux et al., 2017; Gröner et al., 2017), disregarding other contaminant exposure pathways, such as dietary exposure (i.e. trophic transfer along the food chain), which can be particularly important in predatory fish species (Zenker et al., 2014). To the best of our knowledge, so far, only two studies have assessed the ecotoxicological implications of dietary DCF exposure (Guiloski et al., 2015; Ribas et al., 2016). Second, chemical pollution is not the sole environmental stressor that marine species are subject to, and information on the potential effects of other environmental stressors (e.g. seawater warming and acidification) that can particularly affect the bioavailability and toxicity of emerging contaminants is limited (Marques et al., 2010; Amiard-Triquet et al., 2015). So far, only two studies have accounted for the interactive effects of abiotic stressors (both following DCF exposure via water), one using the marine bivalve *Ruditapes philippinarum* under acidification (Munari et al., 2016), and the other using a freshwater fish species *Gasterosteus aculeatus* (adult individuals) under hypoxia (Lubiana et al., 2018). Yet, climate change effects can already be felt in some regions of the world and are expected to worsen in the coming 50–100 years, increasing seawater temperature as much as 5 °C (i.e. ocean warming), as well as increasing CO_2 partial pressure (pCO_2) up to 1000 µatm, which leads to a seawater pH drop (i.e. ocean acidification; IPCC, 2014; McNeil and Sasse, 2016). Thus, gathering data of environmental pollutants in multi-stressors context is urgently needed, as they will provide a better estimate of the potential ecotoxicological implications of pollutants in tomorrow's ocean.

In the field of ecotoxicology, as well as in studies of climate change effects, the marine fish species *Dicenthrachus labrax* has been frequently used as a suitable model species (e.g. Hernández-Moreno et al., 2011; Maulvault et al., 2016, Maulvault et al., 2017; Barboza et al., 2018), given its ecological characteristics and economical value: i) It is a predatory fish species, inhabiting temperate estuaries and coastal areas and likely accumulating high levels of chemical contaminants (FAO, 2018). Therefore, it is a suitable bioindicator in studies following dietary exposure to chemical contaminants. ii) Since *D. labrax* is a commercially valuable species, the deleterious effects of environmental stressors, particularly in its early life stages (including larvae and juveniles) can potentially affect species recruitment and overall ecological success, thus,- certainly having negative impacts in fisheries and aquaculture.

Hence, given the current lack of empirical data on effects of dietary PhAC exposure and the potential interactions of these contaminants with climate change-related stressors, this study aimed to assess, for the first time, different ecotoxicological responses (i.e. animal condition, hematological parameters, genotoxicity, oxidative stress, heat shock response, protein degradation, endocrine disruption and neurotoxicity) induced by dietary DCF exposure (500 ng kg^{-1} dw), seawater warming ($\Delta T^{o}C = 5\,^{\circ}C$) and acidification ($\Delta pCO_2$ ~1000 µatm, equivalent to ΔpH = -0.4 units) in different tissues (brain, liver, muscle and blood) of the European seabass *Dicenthrachus labrax*. Since reaching a general conclusion regarding the severity of stressors can be a challenging process, especially when multiple endpoints and interactive effects of stressors are considered, an "Integrated Biomarker Response" approach (IBR; Guerlet et al., 2010) was used in present study, as its application constitutes a practical and robust tool to be used in field and laboratory studies (e.g. Serafim et al., 2012; Ferreira et al., 2015; Madeira et al., 2016a). Such approach allowed to combine the different biomarker responses observed at the different organization levels (animal, tissue and cell), thus, providing a novel, wider and integrative understanding of the ecological impact of DCF in tomorrow's ocean.

2. Materials and methods

2.1. Control and DCF-contaminated diets

Non-contaminated feed (control, CTR feed) and DCF-contaminated feed (DCF-enriched feed) with the same nutritional composition were manufactured by SPAROS Lda (Olhão, Portugal). Detailed feed composition can be consulted in *Supplementary Materials_Table S1*. Briefly, a control diet (CTR feed) was formulated to mimic a commercial fish-meal-rich formulation for juvenile marine seabass with 62% crude protein and 15% crude fat. All powder ingredients were ground (< 200 µm) using a micropulverizer hammer mill (Hosokawa Micron, SH1, The Netherlands). Ingredients and fish oil were then mixed according to the target formulation in a paddle mixer (Mainca RM90, Spain), and the feed mixture was further humidified with 25% deionized water at room temperature. The diet was extruded at 1.0 mm by means of a low-shear extruder (P55, Italplast, Italy). Upon extrusion, the feed pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). A 10 kg batch of CTR feed was subsequently contaminated with DCF (DCF-enriched feed). To do so, sodium salt of diclofenac ($C_{14}H_{10}C_{l2}NNaO_2$, > 98% purity, Sigma-Aldrich), solubilized in ethanol, was further mixed in fish oil (total volume of 150 mL), and this solution was top-coated to the pellets with a pressurized spraying container (standard flat-fan nozzle; size 250 µm; pressure 2.1 bar). Given the current lack of toxicological data on dietary pharmaceutical exposure, a nominal DCF concentration of approximately 500 ng kg^{-1} on a dry weight basis (dw) was selected to assure that ecotoxicological responses were elicited during the timeline of the trials, corresponding to a value within the range of lowest observed effect concentration for dietary exposure (Guiloski et al., 2015; Ribas et al., 2016) and matching a value ~10 times higher than the DCF concentration commonly found in marine biota inhabiting contaminated coastal areas (e.g. Huerta et al., 2013; Vandermeersch et al., 2015). DCF was subsequently quantified in both control and DCF-enriched diets, following the previously optimized and validated method of Cunha et al. (2017). Final DCF concentration in the DCF-enriched diet was 500 ± 36 ng kg^{-1}, whereas DCF was not detected in the control diet (detection limit in feed = 0.1 ng kg^{-1}; quantification limit in feed = 0.3 ng kg^{-1}).

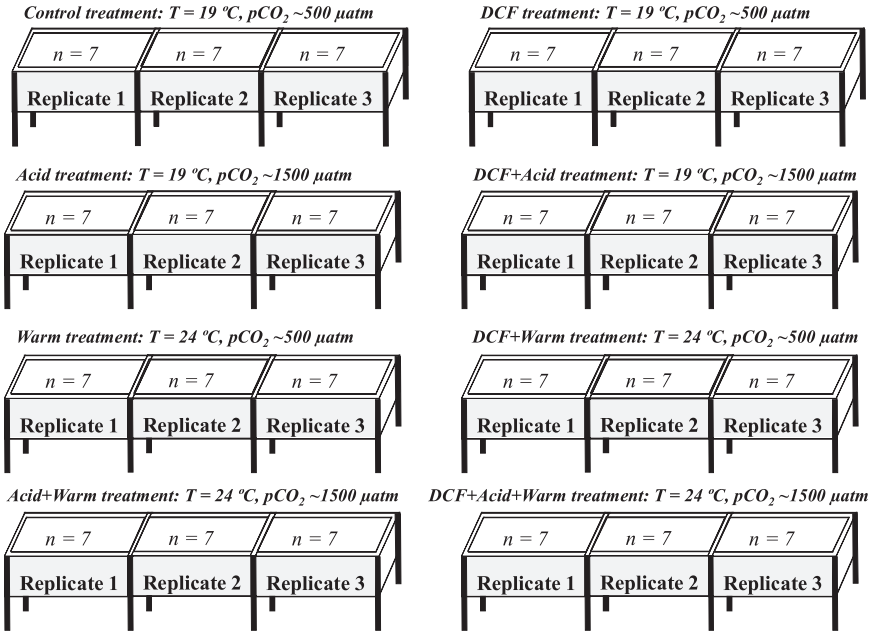
Control treatment: T = 19 ºC, pCO2 ~500 µatm
DCF treatment: T = 19 ºC, pCO2 ~500 µatm
Acid treatment: T = 19 ºC, pCO2 ~1500 µatm
DCF+Acid treatment: T = 19 ºC, pCO2 ~1500 µatm
Warm treatment: T = 24 ºC, pCO2 ~500 µatm
DCF+Warm treatment: T = 24 ºC, pCO2 ~500 µatm
Acid+Warm treatment: T = 24 ºC, pCO2 ~1500 µatm
DCF+Acid+Warm treatment: T = 24 ºC, pCO2 ~1500 µatm
n = 7
Replicate 1
Replicate 2
Replicate 3

Fig. 1. Experimental setup. Abbreviations: DCF – Diclofenac exposure; Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C).

2.2. Organisms and acclimation

D. labrax specimens (n = 168) with similar biometric characteristics were reared until juvenile stage (total length: 6.4 ± 0.4 cm; total weight 4.4 ± 0.2 g) in the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) using routine hatchery conditions. Subsequently, fish were transported to the facilities of "Laboratório Marítimo da Guia (MARE-FCUL, Cascais, Portugal)", where they were randomly but in equal numbers distributed in 24 rectangular-shaped incubating glass tanks (total volume of each 50 L) within independent recirculation aquaculture systems (RAS; i.e. each group of three tanks corresponded to one treatment, and 3 replicate tanks x 8 treatments = 24 tanks in total; Fig. 1; see the description of each treatment in section *2.3.*). Each tank was equipped with protein skimmer (Reef SkimPro, TMC Iberia, Portugal), UV disinfection (Vecton 300, TMC Iberia, Portugal), biological filtration (model FSBF 1500, TMC Iberia, Portugal) and chemical filtration (activated carbon, Fernando Ribeiro Lda, Portugal) to maintain seawater quality. Dead fish and feces were daily removed as well as 25% seawater renewed in each incubation tank. Ammonia, nitrite and nitrate levels were daily checked by means of colorimetric tests (Tropic Marin, USA), and kept below detectable levels, with the exception of nitrates, which were kept below 2.0 mg L^{-1}. Fish density was kept below 5 g body weight L^{-1} in order to avoid physiological stress related to high animal density. Specimens were initially acclimated to laboratory conditions for 30 days, fed with CTR feed (2% average body weight, BW) and kept under abiotic conditions as close as possible to those of their natural habitat: i) dissolved oxygen (DO) > 5 mg L^{-1}; ii) temperature (T ^{0}C) = 19.0 ± 0.5 °C; iii) pH = 8.00 ± 0.10 units; iv) salinity = 35 ± 1‰; and v) photoperiod = 14L:10D (14 h light:10 h dark). Temperature, pH, salinity and DO were daily checked using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany). Temperature and pH were adjusted whenever needed by means of an automatic seawater refrigeration system (± 0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal), as well as submerged digital thermostats (200 W, V2Therm, TMC Iberia, Portugal), and individual pH probes (GHL, Germany) connected to a computerized pH control system (± 0.1 pH units; Profilux 3.1 N, GHL, Germany), which monitored seawater pH in each tank every 2 s, and adjusted whenever needed by injecting CO_2 via submerged air stones (Air Liquide, Portugal; to decrease pH) or by CO_2-filtered aeration (to increase pH) using air pumps (Stella 200, Aqua One Pro, Aqua Pacific UK Ltd, United Kingdom). Total alkalinity of seawater was also measured in every tank on a weekly basis, following the protocol previously described elsewhere (Sarazin et al., 1999) and the combination of total alkalinity (AT) and pH was used to calculate carbonate system parameters (average values obtained for each treatment can be consulted in *Supplementary Materials_Table S.2*). After 30 days of acclimation, 9 fish were randomly sampled in order to determine the baseline levels of each biomarker analyzed, following the procedures described in sections *2.4* and *2.5* (average basal levels can be consulted in *Supplementary Materials_Table S.3*).

2.3. Exposure to DCF, warming and acidification

One week before initiating DCF exposure, seawater temperature and pCO_2 were slowly adjusted (+1 °C and -0.1 pH unit per day), until reaching 24 °C and ~1500 µatm pCO_2 (equivalent to pH = 7.6 units) in tanks simulating climate change conditions (i.e. treatments Acid, Warm, Acid + Warm, DCF + Acid, DCF + Warm and DCF + Acid + Warm; Fig. 1), according to the projections of the Intergovernmental Panel for Climate Change (scenario RPC8.5 of IPCC, 2014; McNeil and Sasse, 2016). The remaining abiotic conditions of the seawater (i.e. DO, salinity and photoperiod) were kept as previously described in section *2.2.* (i.e. as during the acclimation period).

Eight treatments (4 non-contaminated and 4 contaminated; treatments randomly assigned to each tank) were carried out (n = 7 animals per replicate tank of treatment, i.e. a total of 21 animals per treatment; Fig. 1), simulating the reference temperature (i.e. 19 °C) and pCO_2 (~500 µatm; 8.0 pH units) conditions, as well as the projected seawater warming ($\Delta T^{o}C$ = 5 °C) and acidification (Δ pCO_2 ~1000 µatm; equivalent to ΔpH = -0.4 units), using a full cross-factorial design: i) Control treatment, i.e. fish daily fed with CTR feed (2% BW) and exposed to reference temperature and pH conditions; ii) Acid treatment, i.e. fish daily fed with CTR feed (2% BW) and exposed to acidification (1500 µatm pCO_2, equivalent to pH = 7.6); iii) Warm treatment, i.e. fish daily fed with CTR feed (2% BW) and exposed to warming (24 °C); iv) Acid + Warm, i.e. fish daily fed with CTR feed (2% BW) and exposed to warming and acidification (24 °C and ~1500 µatm pCO_2); v)

Table 1
Summary of selected biomarkers and the corresponding methodologies used.

Biomarkers	Category	Tested ecotoxicological response	Type of methodology used	Methodology references
Animal condition (K), hepatosomatic index (HIS) and brain to body mass ratio (BB_{ratio})	Biomarker of effect	Animal fitness and/or disease	Morphometric assessment	Ricker (1975); Diniz et al. (2010)
Blood cell counts	Biomarker of effect	Immune capacity	Optical microscopy	Mumford et al. (2007)
Erythrocyte nuclear anomalies (ENAs)	Biomarker of effect	Genotoxicity	Optical microscopy	Carrasco et al. (1990)
Erythrocyte viability	Biomarker of effect	Citotoxicity	Optical microscopy	Strober (2001)
Catalase (CAT) activity	Biomarker of effect	Oxidative stress	Enzymatic assay	Johansson and Borg (1988); Maulvault et al. (2017)
Superoxide dismutase (SOD) activity	Biomarker of effect	Oxidative stress	Enzymatic assay	Sun et al. (1988); Maulvault et al. (2017)
Glutathione S-transferase (GST) activity	Biomarker of effect	Oxidative stress and xenobiotic detoxification phase II	Enzymatic assay	Habig et al. (1974); Maulvault et al. (2017)
Lipid peroxidation (LPO)	Biomarker of effect	Oxidative stress and cellular damage	TBARS method	Uchiyama and Mihara (1978); Madeira et al. (2016a,2016b)
HSP70/HSC70 proteins content	Biomarker of effect	Chaperoning, heat shock response	Indirect ELISA	Njemini et al. (2005); Maulvault et al. (2017)
Total Ubiquitin (Ub) content	Biomarker of effect	Protein degradation and DNA repair	Direct ELISA	Madeira et al. (2014)
Acetylcholinesterase (AChE) activity	Biomarker of effect and exposure	Neurotoxicity	Enzymatic assay	Ellman et al. (1961); Maulvault et al. (2017)
Vitellogenin (VTG) content	Biomarker of exposure	Endocrine disruption and reproduction	Direct ELISA	Denslow et al. (1999); Diniz et al. (2010)

DCF treatment, i.e. fish daily fed with DCF-enriched feed (2% BW) and exposed to reference temperature and pH conditions; vi) DCF + Acid treatment, i.e. fish daily fed with DCF-enriched feed (2% BW) and exposed to acidification (1500 µatm pCO_2); vii) DCF + Warm treatment, i.e. fish daily fed with DCF-enriched feed (2% BW) and exposed to warming (24 °C); viii) DCF + Acid + Warm treatment, i.e. fish daily fed with DCF-enriched feed (2% BW) and exposed to acidification and warming (24 °C and ~1500 µatm pCO_2). Abiotic seawater parameters were daily checked and adjusted to adequate levels whenever needed as described above. No mortality was observed during the 28-day trial. Throughout the trial, seawater samples were collected from each tank (treatment; days 0, 7, 14 and 28 of the experiment), and DCF concentrations were determined according to the previously optimized and validated method of Cunha et al. (2017). Values were below the detection limit of the method (for water samples = 0.05 µg L^{-1}) in all samples, thus, assuring that no external contamination was taking place in either non-contaminated or in DCF- contaminated treatments (apart from the intended dietary DCF exposure).

2.4. Sample preparation and hematological parameters

By the end of the exposure period, 9 fish were randomly collected from each treatment (i.e. 3 fish collected from each of the 3 replicate tanks that composed one treatment), euthanized by immersion in an overdosed MS222 solution (2000 mg L^{-1}; Sigma-Aldrich, USA) buffered with sodium bicarbonate (1 g of $NaHCO_3$ to 1 g of MS222 to 1 L of seawater) for 10 min. Euthanized fish were measured (total length, TL, and weight, W), and peripheral blood was collected by puncture of the caudal vein.

A fraction of fish blood was immediately used to perform the trypan blue exclusion test of cell viability previously described elsewhere (Strober, 2001). In brief, a 0.4% solution of trypan blue (trypan blue powder reagent, ~40% dye content, Sigma-Aldrich) was prepared in PBS (pH = 7.2–7.3 units) and then 100 µL of blood samples (properly diluted) were added to 100 µL trypan blue in 1.5 mL microtubes. Then, mixed sample was incubated (ca. 3 min), loaded on a Neubauer hemocytometer (HBG, Germany) and analyzed using an optical microscope (OPTIKA B-500, Italy) at a 400× magnification (three observations were made per individual; the average of the three measurements made in each sample/individual was used in statistical analysis).

Fish blood smears were prepared on precleaned glass microscopy slides (three slides per individual), allowed to air-dry, then fixed for 20 min in ethanol (100%) and stained with the ready to use Hemacolor staining reagent (Hemacolor® Rapid staining of blood smear, Sigma-Aldrich) according to the instructions provided in this product, in order to subsequently count blood cells (erythrocytes and leukocytes), as well as to detect the presence of erythrocyte nuclear abnormalities (ENAs) and micronuclei through optical microscopy. Following staining, the microscope glass slides were mounted with DPX (BDH, Poole, England). A minimum of 500 cells per slide were examined under the microscope (1000× magnification; the average of the three slide observations made in each sample/individual was used in statistical analysis). ENAs and micronuclei were counted according to the classification described in Carrasco et al. (1990).

After blood collection, fish were dissected, and fish muscle, brain and liver were collected (approximately 100 mg of muscle and liver, and about 40 mg of brain), homogenized in ice-cold conditions with 1.0 mL of phosphate buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM KH_2PO_4, pH = 7.40 ± 0.02; reagents from Sigma-Aldrich, Germany), using an Ultra-Turrax® device (T25 digital, Ika, Germany). Crude homogenates were centrifuged in 1.5 mL microtubes for 15 min at 10.000 *g* and 4 °C, supernatants were transferred to new microtubes, immediately frozen and kept at -80 °C until further analyses. All biochemical analyses were performed in triplicate using reagents of pro analysis grade or higher.

2.5. Molecular biomarkers

Eight molecular biomarkers (of exposure and/or effect) were selected to assess distinct biological effects (endpoints) induced by DCF, warming and acidification and their co-exposures at the tissue level (Table 1). Table 1 presents a summary of the selected biomarkers, as well as the different methodologies used. These molecular biomarkers have been widely used in ecotoxicological studies, and have previously been considered reliable and suitable to assess the effects of exposures to xenobiotics (for DCF; e.g. Gonzalez-Rey and Bebianno, 2014), as well as climate change-related effects (e.g. Rosa et al., 2016; Jesus et al., 2018; Maulvault et al., 2017). Thus, not only were they considered to fit the purposes of the present study, but also their use enabled comparisons of the present data with previous studies on the ecotoxicological effects of xenobiotics and/or climate change effects. Furthermore, to have a broader view of the effects of the studied stressors in a whole organism context, different fish tissues (brain, liver, muscle and blood) with distinct cell types and susceptibility to stressors were analyzed.

In order to normalize the results of each biomarker (i.e. results presented in mg of protein), total protein levels were also quantified in each sample with the Bradford assay (Bradford, 1976). All protocols used for both enzymatic and protein quantification assays were adapted to 96-well microplates, and were validated and described in detail in earlier reports (e.g. Diniz et al., 2010; Madeira et al., 2016a; Maulvault et al., 2017). In all methodologies, 96-well microplates from Nunc-Roskilde (Denmark) as well as a microplate reader (BioRad, Benchmark, USA) were used.

2.6. Animal fitness indexes (K, HSI and BB_{ratio}) and integrated biomarker responses (IBR)

The Fulton's K index was directly calculated from the biometric data to determine fish condition, according to the formula:

$$K = 100 \times \frac{W\ (g)}{TL^3(cm)}$$

where W is the fish weight and TL is the total length (Ricker, 1975). The relationship between fish total weight and the respective organ weight was calculated for liver (i.e. hepatosomatic index, HSI) and brain (i.e. brain to body mass ratio, BB_{ratio}) to provide information on liver and brain condition, using the following equations:

$$HSI = \frac{Liver\ weight\ (g)}{W\ (g)} \times 100$$

and,

$$BB_{ratio} = \frac{brain\ weight\ (g)}{W\ (g)} \times 100$$

To better understand and relate the ecotoxicological effects induced by each experimental treatment, biomarkers related to animal fitness in a whole organism context (i.e. K, HSI, BB_{ratio} and hematological parameters), as well as molecular biomarkers in the different fish tissues (i.e. CAT, SOD, GST, LPO and AChE activities and HSP70/HSC70, Ub and VTG contents) were integrated by calculating the "Integrated biomarker response" (IBR) for each treatment (and tissue, for molecular biomarkers), according to the methodology proposed by Beliaeff and Burgeot (2002), later modified by Guerlet et al. (2010) (full details regarding the IBR methodology are available in these earlier reports as well as in Serafim et al., 2012; Ferreira et al., 2015; Madeira et al., 2016a). As IBR compares biomarker responses of organisms exposed to stressors to those of animals under control conditions (or their baseline levels), in general, lower biomarker scores (and, thus, lower IBR index values) are indicative of a better health state (higher animal fitness), whereas higher scores usually indicate that organisms are in a poorer physiological condition (i.e. stressed; e.g. Serafim et al., 2012; Ferreira et al., 2015; Madeira et al., 2016a). Star plots and IBR calculations were performed using the Microsoft Excel software.

2.7. Statistical analysis

As standard procedure, data were first tested for normality and homoscedasticity through Kolmogorov–Smirnov and Levene tests, respectively. Data were log-transformed or square-rooted, whenever at least one of these assumptions of ANOVA was not verified. To evaluate the presence of significant differences between treatments in fish morphometric data (W and TL), hematological parameters (percentage of erythrocytes, leukocytes, ENAs, micronuclei and viable erythrocytes in relation to total blood cells or total erythrocyte counts), brain AChE activity and liver VTG content, one-way nested-ANOVA analysis was performed (with replicate tank as nesting factor), whereas for the remaining biochemical biomarkers (CAT, SOD, GST, LPO, HSP70/HSC70 and Ub), two-way ANOVAs were carried out instead, using tissue (brain, liver and muscle) and treatment (non-contaminated/DCF contamination, reference temperature/warming and reference pH/acidification) as variables. The existence of significant differences between whole organism and tissue IBRs were also analyzed using a simple one-way ANOVA. Finally, differences in animal fitness indexes were tested using an ANCOVA analysis, with W and TL as co-variates of K, and W as co-variate of HSI and BB_{ratio}. Post-hoc Tukey HSD tests were subsequently conducted to identify significant differences. Statistical analyses were performed at a significance level of 0.05, using STATISTICA™ software (Version 7.0, StatSoft Inc., USA).

3. Results

3.1. Fish morphometry and condition

Although higher TL and W were found in Control fish after 28 days of exposure compared to the baseline values, similar animal condition (K), hepatosomatic index (HSI) and brain to body mass ratio (BB_{ratio})

Table 2
Total length (TL; cm) and weight (W; g), Fulton's condition index (K), hepatosomatic index (HIS) and brain-to-body mass ratio (BB_{ratio}) in *D. labrax* after 28 days of exposure (mean ± SD; $n = 9$). In each column, different letters indicate significant differences ($p < 0.05$) between treatments. Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0); DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6); Warm – simulated warming (i.e. T = 24 ºC).

	TL	W	K	HSI	BB_{ratio}
Control	7.7 ± 0.2^{bc}	8.9 ± 0.7^{ab}	1.94 ± 0.10^{a}	2.41 ± 0.18^{a}	1.05 ± 0.08^{a}
Acid	8.2 ± 0.2^{ab}	10.1 ± 0.6^{a}	1.86 ± 0.01^{ab}	1.97 ± 0.32^{ab}	0.56 ± 0.05^{c}
Warm	8.4 ± 0.2^{a}	10.7 ± 1.5^{a}	1.78 ± 0.15^{abc}	2.17 ± 0.58^{ab}	0.78 ± 0.04^{b}
Acid + Warm	8.2 ± 0.5^{ab}	10.3 ± 1.2^{a}	1.88 ± 0.15^{a}	2.03 ± 0.33^{ab}	0.59 ± 0.01^{c}
DCF	7.3 ± 0.6^{c}	7.3 ± 1.5^{b}	1.87 ± 0.11^{a}	1.08 ± 0.42^{c}	0.79 ± 0.13^{b}
DCF + Acid	8.1 ± 0.4^{ab}	9.7 ± 1.2^{a}	1.82 ± 0.01^{abc}	2.28 ± 0.72^{ab}	0.74 ± 0.11^{b}
DCF + Warm	8.2 ± 0.3^{ab}	9.4 ± 0.8^{a}	1.70 ± 0.07^{c}	1.70 ± 0.53^{abc}	0.75 ± 0.12^{b}
DCF + Acid + Warm	8.0 ± 0.3^{ab}	9.0 ± 1.0^{ab}	1.75 ± 0.02^{bc}	1.60 ± 0.19^{bc}	0.86 ± 0.04^{b}

were observed (Table 2 and *Supplementary Materials_Table S3)*. Comparing with results observed under the reference temperature and pCO_2 conditions (Control treatment), warming promoted a significant increase in animal TL (Table 2; $p = 0.02$). On the other hand, fish from DCF treatment exhibited significantly lower W than those exposed to warming and/or acidification (i.e. $p = 0.007$, $p = 0.002$, $p = 0.005$, $p = 0.021$ and $p = 0.039$ in Acid, Warm, Acid + Warm, DCF + Acid and DCF + Warm treatments, respectively), but not compared to Control treatment ($p = 0.115$; Table 2). Regarding K, despite the general decrease promoted by the three stressors (alone or combined) compared to the Control treatment, significant differences were only observed when warming was combined with DCF, regardless of pCO_2 conditions (corresponding to a 12% and 10% decrease in DCF + Warm and DCF + Acid + Warm, respectively, in relation to the Control treatment; $p = 0.006$ and $p = 0.024$, respectively; Table 2, as well as *Supplementary Materials_Supplementary Materials_Table S4*). Overall, DCF, warming and/or acidification decreased fish liver size, with fish exposed to DCF alone or to the three stressors simultaneously (i.e. DCF + Acid + Warm treatment) exhibiting significantly lower HSI than non-contaminated samples, regardless of temperature and pCO_2 conditions (equivalent to a 55% and 34% decrease in relation to Control treatment, respectively; $p = 0.001$ in both cases; Table 2 and *Supplementary Materials_Supplementary Materials_Table S4*). Interestingly, DCF alone also yielded significantly lower HSI than DCF and acidification co-exposure (i.e. treatment DCF + Acid; $p = 0.005$; Table 2). All stressors acting alone or in combination promoted a significant reduction of the BB_{ratio} (equivalent to a decrease of 18% to 47% in DCF + Acid + Warm and Acid treatments, respectively, compared to the Control; $p = 0.003$ and $p = 0.001$, respectively; Table 2 and *Supplementary Materials_Supplementary Materials_Table S4*). In addition, non-contaminated fish exposed to acidification (at both temperature exposures; i.e. Acid and Acid + Warm treatments) also revealed significantly lower BB_{ratio} than non-contaminated fish exposed to warming alone (i.e. Warm treatment; $p = 0.008$; Table 2).

3.2. Hematological parameters

Similar hematological parameters were found in the baseline and Control groups (Table 3 and *Supplementary Materials_Table S3)*. Significantly different percentages of erythrocytes (lower; $p = 0.047$) and leukocytes (higher; $p = 0.046$) were found in non-contaminated fish under acidification and warming co-exposure compared to the remaining treatments, with the exception of DCF + Warm treatment ($p > 0.050$; Table 3). DCF-exposed fish exhibited significantly higher percentages of erythrocyte nuclear abnormalities (ENAs) compared to non-contaminated fish, regardless of temperature and pCO_2 levels (between 27.1% and 33.7% in DCF + Acid + Warm and DCF treatments, respectively; $p = 0.040$ and $p = 0.002$, respectively; Table 3 and *Supplementary Materials_Table S3*). Noteworthy, in general, no micronuclei (i.e. the most severe form of ENA) were observed in non-contaminated fish (except in two samples of the Acid treatment, corresponding to an average of 1.5%), whereas up to 12.5% of micronuclei where found in contaminated fish (i.e. maximum number of micronuclei observed in DCF-treatment; $p < 0.0001$; Table 3). Similarly, erythrocyte viability was reduced by all stressors, except by warming acting alone ($p > 0.050$), yet, the lowest values were observed in fish under the co-exposure of DCF and acidification, regardless of temperature, (averages ranging from 32.9% to 39.8%; Table 3; $p = 0.001$ in both cases; Table 3).

3.3. Antioxidant defenses and lipid peroxidation

Basal antioxidant biomarker levels were similar to those in the Control treatment (Figs. 2 and 3A–C and *Supplementary Materials_Table S3)*. Overall, different patterns were observed according to tissue and treatment. CAT activity significantly varied among tissues of contaminated fish (being higher in the liver followed by brain and muscle; $p < 0.050$), but not in non-contaminated tissues (Fig. 2A–C). In fish muscle, DCF exposure inhibited CAT activity (between 57 and 80%, in DCF + Warm and DCF treatments, respectively; $p = 0.046$ and $p = 0.007$, respectively), regardless of temperature and pCO_2 exposure Fig. 2A, as well as *Supplementary Materials_Supplementary Materials_Table S4*). In liver, although values in Control treatment were not significantly different from those in the remaining treatments, DCF exposure alone resulted in significantly higher CAT activity than when combined with acidification ($p = 0.001$ and $p = 0.047$ in DCF + Acid and DCF + Acid + Warm treatments, respectively; Fig. 2B). As for the brain, no significant differences were found between treatments ($p > 0.050$; Fig. 2C).

Concerning SOD, higher inhibition was observed in fish brain and liver (between 63.1% and 78.5%) compared to muscle (between 39.3% and 72.1%), regardless of treatment ($p < 0.05$; Fig. 2D–F). In fish muscle, DCF exposure, warming and acidification drastically increased SOD inhibition, with the highest values being observed in non-contaminated acidified treatments, corresponding to an average increase of 58–67% in relation to the Control treatment ($p < 0.0001$ in Acid and Acid + Warm treatments; Fig. 2D, as well as *Supplementary Materials_Supplementary Materials_Table S4*). Noteworthy, higher SOD inhibition percentages were found in non-contaminated muscle ($p < 0.0001$; Fig. 2D). A different trend was observed in the liver, as fish exposed to acidification and DCF (alone or combined) showed significantly lower SOD inhibition than Control fish (i.e. a reduction of 9–11%; $p < 0.0001$ in Acid, DCF and DCF + Acid treatments; Fig. 2E, as well as *Supplementary Materials_Supplementary Materials_Table S4*). As for fish brain, overall, SOD activity did not vary among treatments, with the exception of DCF exposure combined with acidification (i.e. 70.3% of SOD inhibition), which yielded significantly lower inhibition compared to DCF exposure alone (i.e. 75.8% of SOD inhibition; $p = 0.021$; Fig. 2F).

Overall, fish liver exhibited higher GST activity than the muscle and

Table 3
Percentage of erythrocytes and leukocytes in relation to total cell counts, as well as percentage of viable erythrocytes (Viable Ery), erythrocyte nuclear abnormalities (ENAs) and micronuclei in relation to total erythrocyte counts (mean ± SD; $n = 9$). In each column, different letters indicate significant differences ($p < 0.05$) between treatments. Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0); DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6); Warm – simulated warming (i.e. T = 24 ºC).

	Erythrocytes	Leucocytes	ENAs	Micronuclei	Viable Ery
Control	95.6 ± 1.2^{a}	4.4 ± 1.2^{b}	11.5 ± 6.8^{b}	0.0 ± 0.0^{c}	76.2 ± 3.4^{a}
Acid	98.2 ± 0.7^{a}	1.8 ± 0.7^{b}	9.5 ± 1.7^{b}	1.5 ± 2.6^{bc}	64.5 ± 2.6^{b}
Warm	96.9 ± 1.1^{a}	3.1 ± 1.1^{b}	12.1 ± 3.9^{b}	0.0 ± 0.0^{c}	76.4 ± 2.5^{a}
Acid + Warm	90.3 ± 4.5^{b}	9.7 ± 4.5^{a}	15.1 ± 1.9^{b}	0.0 ± 0.0^{c}	68.0 ± 3.0^{b}
DCF	97.0 ± 0.5^{a}	3.0 ± 0.5^{b}	33.7 ± 4.9^{a}	8.0 ± 4.0^{a}	47.7 ± 2.0^{c}
DCF + Acid	98.2 ± 0.9^{a}	1.8 ± 0.9^{b}	31.3 ± 7.7^{a}	5.0 ± 4.4^{ab}	39.8 ± 3.3^{de}
DCF + Warm	95.4 ± 0.6^{ab}	4.6 ± 0.6^{ab}	30.3 ± 5.8^{a}	4.9 ± 3.9^{ab}	45.4 ± 5.9^{cd}
DCF + Acid + Warm	97.4 ± 1.5^{a}	2.6 ± 1.5^{b}	27.1 ± 6.4^{a}	5.9 ± 5.2^{ab}	32.9 ± 2.2^{e}

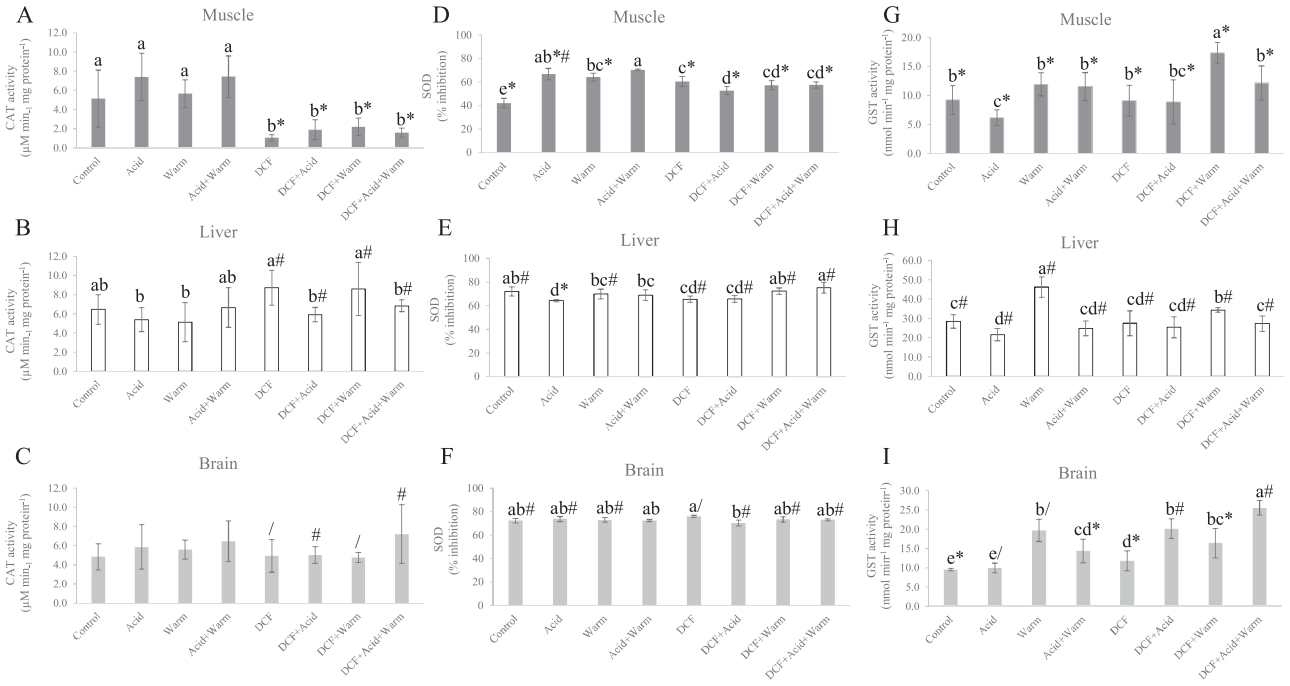

Fig. 2. Anti-oxidant enzyme activity (GST, nmol min $^{-1}$ mg^{-1} protein; CAT, μM min $^{-1}$ mg^{-1} protein; SOD, % inhibition) in muscle (A, D, G), liver (B, E, H) and brain (C, F, I) of *D. labrax* after 28 days of exposure (mean ± SD; $n = 9$). Different letters indicate significant differences between treatments ($p < 0.05$), whereas different symbols (*, # and /) indicate significant differences between tissues in the same treatment. Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0); DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6); Warm – simulated warming (i.e. T = 24 °), CAT – catalase activity; SOD – superoxide dismutase inhibition; GST – glutathione S-transferase.

brain, with values ranging between 19.0 and 51.7 nmol min^{-1} mg protein^{-1} in this tissue ($p < 0.050$; Fig. 2G–I). In fish muscle, DCF exposure combined with warming resulted in significantly higher GST activity compared to the other treatments (corresponding to an increase of 88% in relation to the Control treatment; $p < 0.0001$; Fig. 2G, as well as *Supplementary Materials_Supplementary Materials_Table S4*). Conversely, acidification induced the opposite effect, resulting in an average reduction of 33% in relation to the Control treatment ($p = 0.022$; Fig. 2G, as well as *Supplementary Materials_Supplementary Materials_Table S4*). A similar trend was found in fish liver, with Acid treatment also showing an impairment of GST activity in relation to the Control treatment (i.e. 24% of average reduction; $p = 0.008$), yet, warming alone was responsible for the highest induction of this enzyme (corresponding to an increase of 63% in Warm treatment in relation to the Control treatment; $p = 0.012$; Fig. 2H, as well as *Supplementary Materials_Supplementary Materials_Table S4*). In the brain, with the exception of Acid treatment, all treatments induced GST activity compared to Control conditions, but the highest induction was observed when the three stressors were combined (i.e. in DCF + Acid + Warm treatment; $p < 0.0001$; Fig. 2I, as well as *Supplementary Materials_Supplementary Materials_Table S4*).

Lipid peroxidation (measured as MDA concentration) in tissues from each treatment is shown in Fig. 3. In general, higher LPO was observed in brain and muscle compared to liver ($p < 0.050$; Fig. 3). As for differences among treatments, in muscle, significantly higher LPO was observed in non-contaminated fish under warmer conditions ($p = 0.009$ in Warm and $p < 0.0001$ Acid + Warm treatments, respectively), regardless of pCO_2 levels, as well as in DCF + Warm treatment ($p = 0.023$; Fig. 3A and *Supplementary Materials_Supplementary Materials_Table S4*). On the other hand, DCF exposure combined with acidification (regardless of temperature) yielded lower LPO values than those observed in the Control treatment (equivalent to an average decrease of 30% and 48% in DCF + Acid and DCF + Acid + Warm treatments, respectively; $p = 0.027$ and $p = 0.030$, respectively; Fig. 3A and *Supplementary Materials_Supplementary Materials_Table S4*). In liver and brain, none of the treatments simulating DCF exposure, acidification and/or warming was significantly different from the Control treatment (Fig. 3B and C). However, in fish brain, significantly higher LPO was observed in Acid + Warm and DCF treatments compared to treatments simulating the stressors co-exposure (i.e. $p = 0.042$, $p = 0.034$ and $p = 0.018$ in DCF + Acid, DCF + Warm and DCF + Acid + Warm treatments, respectively; Fig. 3C).

3.4. Chaperoning and protein degradation

Basal HSP70/HSC70 and Ub protein contents were similar to those in the Control treatment (Fig. 3D–I, and *Supplementary Materials_Table S3)*. In general, higher concentrations of HSP70/HSC70 proteins were found in fish muscle and brain compared to liver ($p < 0.050$; Fig. 3D–F). In comparison with the Control treatment, significantly higher levels of these proteins were observed in the muscle of contaminated fish under acidification or warming alone (i.e. an average increase of 44% and 84% in treatments DCF + Acid and DCF + Warm, respectively; $p = 0.033$ and $p < 0.0001$, respectively; Fig. 3D and *Supplementary Materials_Supplementary Materials_Table S4*). In contrast, HSP70/HSC70 protein synthesis in the muscle was impaired under acidification alone (i.e. an average reduction of 24% in Acid treatment in relation to the Control treatment; $p = 0.038$; Fig. 3D and *Supplementary Materials_Supplementary Materials_Table S4*). Increased temperatures resulted in significantly higher HSP70/HSC70 protein concentrations in the liver of non-contaminated fish, but not in DCF-exposed ones (equivalent to a 56% and 67% increase in Warm and Acid + Warm treatments, respectively; $p = 0.019$ and $p = 0.006$, respectively; Fig. 3E and *Supplementary Materials_Supplementary Materials_Table S4*). A different pattern was observed in fish brain, with HSP70/HSC70 protein concentrations being significantly higher in Acid, Warm and DCF + Warm treatments compared to the Control treatment (i.e. average increases of 28%, 50% and 33% in relation to Control, respectively; $p = 0.043$, $p = 0.009$ and $p = 0.047$, respectively), but significantly lower in DCF and DCF + Acid + Warm

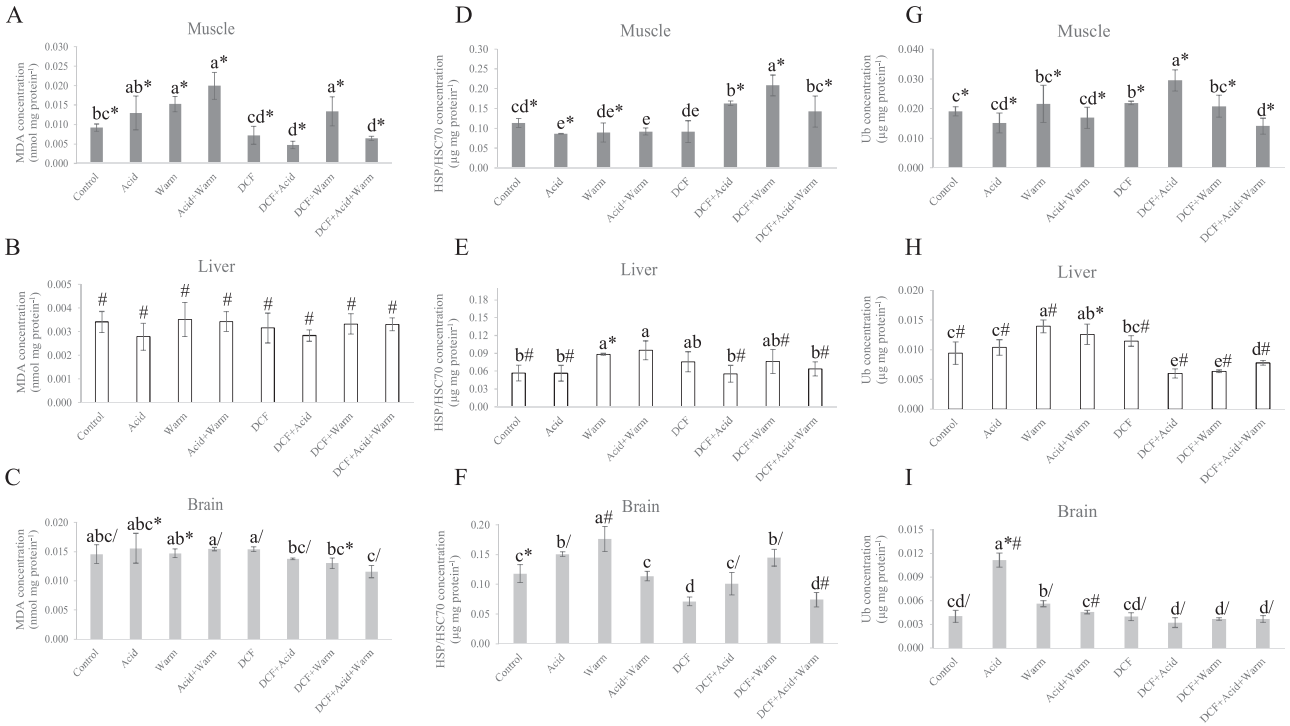

Fig. 3. Lipid peroxidation (expressed as MDA concentration; nmol mg^{-1} protein), HSP/HSC70 concentration (µg mg^{-1} protein), and ubiquitin concentration (Ub; µg mg^{-1} protein) in muscle (A, D, G), liver (B, E, H) and brain (C, F, I) of *D. labrax* after 28 days of exposure (mean ± SD; $n = 9$). Different letters indicate significant differences between treatments ($p < 0.05$), whereas different symbols (*, # and /) indicate significant differences between tissues in the same treatment. Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ºC and pH = 8.0); DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6); Warm – simulated warming (i.e. T = 24 °C); MDA –lipid peroxidation measured as malondialdehyde concentration; HSP70/HSC70 – heat shock proteins; Ub – total ubiquitin.

treatments (i.e. average decrease of 40%; $p = 0.012$ and $p = 0.025$, respectively; Fig. 3F and *Supplementary Materials_Supplementary Materials_Table S4*).

In general, higher Ub concentration was found in fish muscle compared to the liver and brain ($p < 0.050$; Fig. 3G–I). In comparison with the Control treatment, DCF alone or in co-exposure with acidification promoted significantly higher Ub concentrations in the muscle (i.e. an average synthesis induction of 15% and 55% in DCF and DCF + Acid treatments, respectively; $p = 0.041$ and $p = 0.006$, respectively; Fig. 3G and *Supplementary Materials_Supplementary Materials_Table S4*). Conversely, the combination of the three stressors inhibited the synthesis of Ub in the muscle, resulting in an average decrease of 26% in relation to the Control treatment (i.e. in DCF + Acid + Warm treatment; $p = 0.022$; Fig. 3G and *Supplementary Materials_Supplementary Materials_Table S4*). In liver, while warming (with or without acidification) enhanced Ub concentration (48% and 34% increase in relation to Control treatment; $p = 0.008$ and $p = 0.020$, respectively), DCF exposure combined with acidification and/or warming elicited the opposite effect (a reduction of 36%, 33% and 18% in DCF + Acid, DCF + Warm and DCF + Acid + Warm treatments, respectively; $p = 0.025$, $p = 0.039$ and $p = 0.043$, respectively; Fig. 3H and *Supplementary Materials_Supplementary Materials_Table S4*). As for fish brain, acidification and warming acting alone increased Ub concentration (i.e. average increase of 40% and over 100% in Warm and Acid treatments, respectively, compared to the Control treatment; $p = 0.007$ and $p < 0.0001$, respectively), while in the remaining treatments, concentrations did not significantly differ from those observed in the Control treatment ($p > 0.050$; Fig. 3I and *Supplementary Materials_Supplementary Materials_Table S4*).

3.5. Neurotoxicity and endocrine disruption

Basal AChE brain activity and VTG content were similar to those in the Control treatment (Fig. 4 and *Supplementary Materials_Table S3)*. In non-contaminated fish, warming acting alone resulted in a significantly higher AChE activity (i.e. in Warm treatment) compared to treatments simulating acidification (regardless of temperature exposure; $p = 0.041$ and $p = 0.031$ in Acid and Acid + Warm treatments, respectively), but not compared to the Control treatment (Fig. 4A). In DCF-exposed fish, increased AChE activity was observed under the reference temperature and pCO_2 conditions (i.e. in DCF treatment), or when the three stressors were acting together (i.e. in DCF + Acid + Warm), compared to acidification and warming acting alone (i.e. $p = 0.033$ and $p = 0.015$ in DCF + Acid and DCF + Warm treatments respectively; Fig. 4A). Noteworthy, DCF and DCF + Warm treatments were also significantly different from the Control treatment (17% higher and 13% lower, respectively, $p = 0.033$ and $p = 0.041$, respectively; Fig. 4A and *Supplementary Materials_Supplementary Materials_Table S4*).

Concerning VTG content in liver, fish exposed to warming (regardless of pCO_2 levels and DCF) generally had significantly higher VTG content than those exposed to the control temperature (i.e. $p = 0.013$, $p = 0.016$ and $p = 0.033$ in Warm, Acid + Warm, and DCF + Warm, respectively), whereas significantly lower values were observed under increased pCO_2 levels ($p > 0.050$; Fig. 4B and *Supplementary Materials_Supplementary Materials_Table S4*). It is also worth noting that DCF exposure acting alone also significantly increased VTG concentration in relation to the levels found in the Control treatment (equivalent to 87% of increase in DCF treatment; $p < 0.0001$; Fig. 4B and *Supplementary Materials_Supplementary Materials_Table S4*).

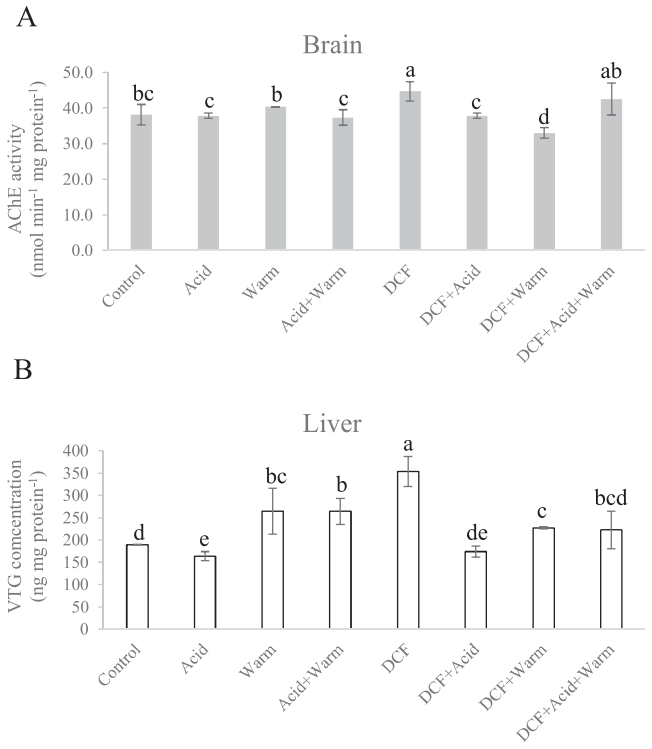

Fig. 4. Acetylcholinesterase (AChE) activity (nmol min $^{-1}$ mg^{-1} protein; 4 A) in brain tissues and vitellogenin (VTG) concentration (ng mg^{-1} protein; 4B) in liver tissues of *D. labrax* after 28 days of exposure (mean ± SD; $n = 9$). Different letters indicate significant differences between treatments ($p < 0.05$). Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 ° and pH = 8.0); DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6); Warm – simulated warming (i.e. T = 24 °); AChE – acetylcholinesterase activity; VTG – vitellogenin content.

3.6. Integrated biomarker responses (IBRs)

The starplots combining IBRs of the different biomarkers are presented in Fig. 5 (individual biomarker scores can also be consulted in *Supplementary Materials_Table S5*). Fish liver and brain were more responsive than muscle and whole organism (all treatments combined; One-way ANOVA, F = 6.1126; $p =$ 0.0025; Fig. 5A). Furthermore, the different treatments promoted distinct biomarker responses in the whole organism, muscle, liver and brain (Fig. 5 and *Supplementary Materials_Table S5*). In terms of biomarkers assessed from a whole organism perspective, the combination of DCF exposure with climate change effects, particularly acidification, yielded the highest IBR index value with hematological parameters (erythrocyte:leukocyte total count ratio, % ENAs and viable erythrocytes) being the most responsive biomarkers (i.e. highest scores), whereas the Control treatment resulted in the lowest (Fig. 5A–E and *Supplementary Materials_Table S5*). Control treatment also resulted in the lowest IBR index value in muscle and brain, but not in liver (Fig. 5A, F–Q and *Supplementary Materials_Table S5*). In fish muscle, the highest IBR was attributed to the combination of DCF exposure and warming, with higher biomarker scores obtained for SOD, GST and HSP70/HSC70 in this treatment (Fig. 5A, F–I and *Supplementary Materials_Table S5*). Warming alone resulted in the highest IBR index value in the liver, with GST, HSP70/HSC70 and Ub yielding the most contributing scores (Fig. 5A, J–M and *Supplementary Materials_Table S5*). Noteworthy, acidification (regardless of DCF exposure) revealed the lowest IBR index values in fish liver (Fig. 5A, J–M and *Supplementary Materials_Table S5*). In the brain, DCF exposure alone showed the highest IBR index value, predominated by the scores of CAT, SOD and AChE activities (Fig. 5A, N–Q and *Supplementary Materials_Table S5*).

4. Discussion

Studies assessing the interactions between chemical contaminants and climate change-related stressors are still very few, both in field or laboratory conditions. Yet, the distinct trends observed according to treatment, biomarker and tissue highlighted the importance, not only of analysing different biomarkers/tissues to have a broader view of stressors in a whole organism context, but also of considering the potential interactions between multiple stressors in ecotoxicological studies.

4.1. General animal fitness

As observed in the present study (i.e. higher TL in Warm treatment), warmer temperatures are usually associated with an enhanced animal growth in ectotherms (e.g. Maulvault et al., 2017; Sandblom et al., 2016). However, animal fitness can also gradually decline when temperature is beyond optimal temperature range for a species or when additional stresses take place simultaneously, either implying additional energetic costs (Pörtner and Peck, 2010) or preventing the allocation of energy resources towards somatic growth (Pistevos et al., 2015; Sandblom et al., 2016; Maulvault et al., 2017; Anacleto et al., 2018), thus explaining the reduced animal condition (i.e. lower K) observed, when warming was combined with DCF exposure and acidification.

Although the exposure to chemical contaminants has been frequently linked to liver hypertrophy and/or hyperplasia (Diniz et al., 2009; Sadekarpawar and Parikh, 2013; Maulvault et al., 2017), a different trend was observed in this study, as DCF exposure alone or the combination of the three studied stressors resulted in an HSI reduction. A similar result was also reported for *Hoplias malabaricus* exposed to DCF from dietary sources (Guiloski et al., 2015). Such reduction could be due to: i) tissue damage induced by DCF (Triebskorn et al., 2004; Pandey et al., 2017), which could have been reversed by warming and acidification acting separately (i.e. DCF + Acid and DCF + Warm); and/or ii) severe metabolic changes induced by the combination DCF, warming and acidification (explaining the lower K registered in this treatment), which could have interfered with the deposition of glycogen in the liver and with the stability of structural components, such as lipids and proteins, thus affecting liver weight (Triebskorn et al., 2004; Anacleto et al., 2018).

The three stressors substantially decreased brain mass in relation to body weight, probably due to a reduction in the number of brain cells (i.e. cell death elicited by these stressors), which can likely result in cognitive impairments. Acidification (alone or in co-exposure with warming) led to the greatest brain mass reduction, and such result could be linked to CO_2 acid-base balance disturbances and to the osmoregulatory mechanisms that are triggered under increased pCO_2 levels (Nilsson et al., 2012; Heuer et al., 2016). Although research on fish neurophysiology and its potential links with environmental stressors is still on its infancy, recent studies have demonstrated that both pollutants and climate change-related stressors (particularly acidification) can substantially alter fish behavior and cognition and, therefore, lead to dramatic ecological consequences (Nilsson et al., 2012; Lopes et al., 2016; Heuer et al., 2016; Maulvault et al., 2018).

4.2. Immune capacity and genotoxicity

Fish immune system can be either stimulated or suppressed by the exposure to xenobiotics (e.g. Bado-Nilles et al., 2009; Ribas et al., 2016), and hostile abiotic conditions can further immuno-compromise fish health (Chiaramonte et al., 2016). Moreover, the immunological and health-state changes induced by environmental stressors have been previously associated to increased fish susceptibility to disease and, ultimately, to the decline of fish populations in the wild (e.g. Kennedy and Farrell, 2008; Sueiro and Palacios, 2016). Yet, contrasting to the

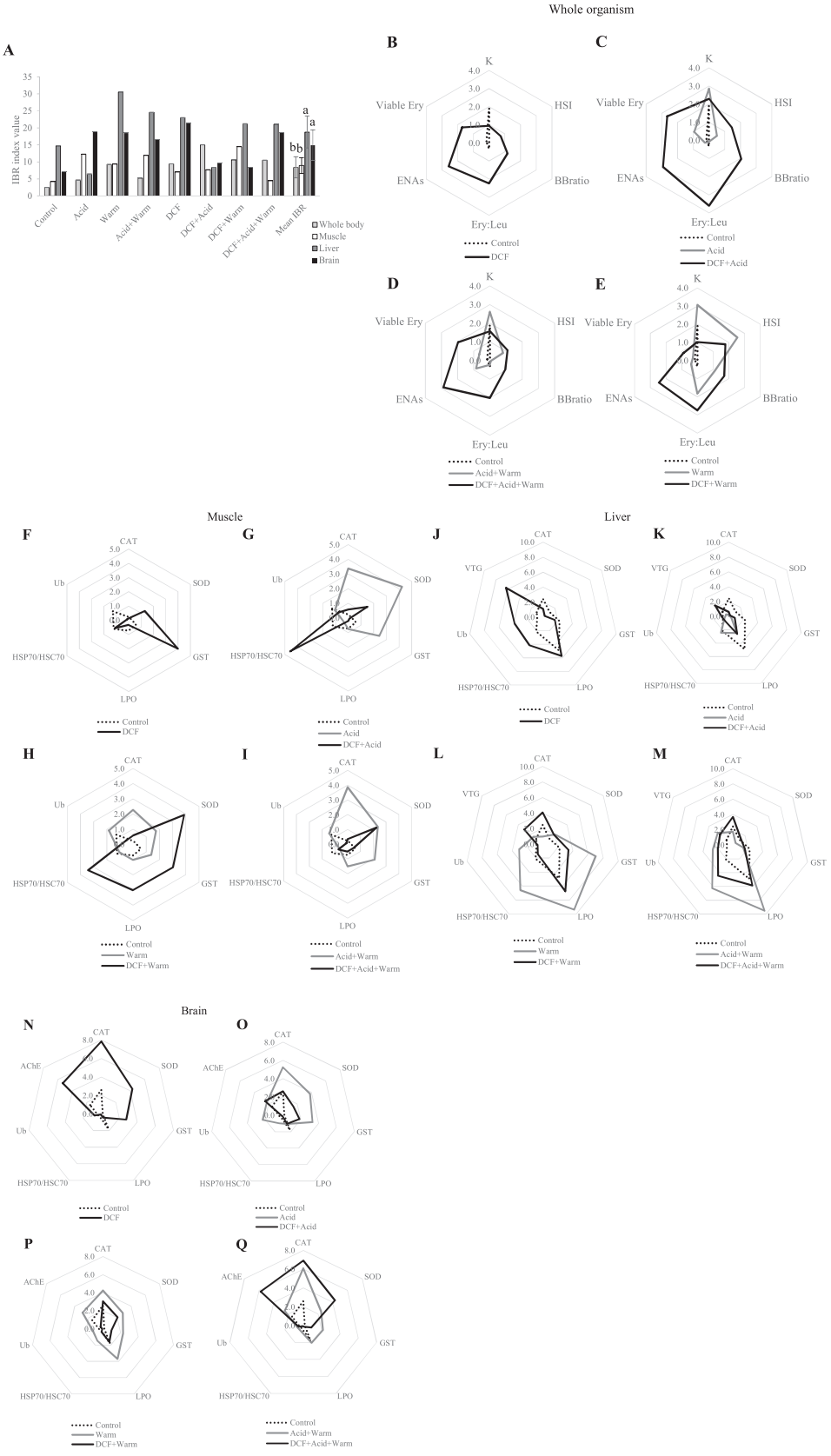

Fig. 5. Total IBR index value and mean IBR (± standard deviation; all treatments combined; 5 A), as well as, star plots for each treatment, including the different biomarkers analysed in the whole organism (5B – 5E) and in the different fish tissues (muscle: 5 F – 5I; liver: 5J–5 M; brain: 5 N – 5Q). Fig. 5A, different letters indicate significant differences in mean IBR ($p < 0.05$). Abbreviations: K - Fulton's condition index; HIS - hepatosomatic index; BB_{ratio} - brain-to-body mass ratio, Ery:Leu – ratio between total erythrocyte and total leukocyte counts; ENAs - erythrocyte nuclear abnormalities (including micronuclei); Viable Ery - viable erythrocytes; CAT – catalase; SOD – superoxide dismutase; GST – glutathione S-transferase; LPO - lipid peroxidation; HSP70/HSC70 - heat shock proteins; Ub - total ubiquitin; AChE – acetylcholinesterase; VTG – vitellogenin; DCF – DCF exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C).

results reported by Saravanan et al. (2011), Ribas et al. (2016) and Mathias et al. (2018) in freshwater fish species *Cyprinus carpio*, *Hoplias malabaricus* and *Rhamdia quelen*, respectively, no significant alterations in erythrocyte and leukocyte counts were elicited by DCF in the present study. This could be related to a dose-dependent action of NSAIDs, such as DCF, or to distinct compound bioavailability according to the uptake pathway (i.e. water exposure in Saravanan et al., 2011; or trophic transfer through the ingestion of preys previously injected with DCF in

Ribas et al., 2016). As for the effect of abiotic stressors, although the stress induced by the combination of warming and acidification (i.e. Acid + Warm treatment) resulted in increased leukocyte counts, such immunostimulation seemed to have been reversed by DCF (i.e. DCF + Acid + Warm treatment), most likely due to the anti-inflammatory action of this compound. In this way, while temperature changes have been previously described to have a suppressive effect on fish immune system (Magnadóttir, 2006), further research is needed in what concerns the effects of seawater pH levels and the interactive effects with other stressors on fish immunity.

All stressors, except warming acting solely, significantly decreased erythrocyte viability, and this result not only evidenced the cytotoxicity of DCF, but also suggested that the deleterious effects of DCF on fish immune capacity were enhanced by warming and acidification. Moreover, the frequency of observed ENAs, particularly, in its most severe form (i.e. micronuclei) was substantially increased by DCF exposure, regardless of temperature and pCO_2 conditions, therefore, evidencing DCF's genotoxic effects. Research on the immunological and genotoxicological effects of NSAIDs in marine fish species is extremely limited and, to the best of the authors' knowledge, the interactive effects of these compounds with abiotic variables have not been previously considered thus hindering comparisons with the current data. Nonetheless, recent studies on mollusks (e.g. *Dreissena polymorpha,* Parolini et al., 2011; and *Lymnaea stagnalis,* Boisseaux et al., 2017) did not find remarkable cyto-genotoxic effects following short-term exposure to DCF. Similarly, Guiloski et al. (2017) found no significant differences in the number of micronuclei between control and DCF-exposed groups in the freshwater fish *Rhamdia quelen*. As argued by Boisseaux et al. (2017), such absence of cyto-genotoxic effects in these studies may be due to different reasons, such as the exposure duration (short-term exposures were carried out in Parolini et al., 2011 and Boisseaux et al., 2017), contaminant concentration or exposure pathway (DCF exposure through water in Parolini et al., 2011; Boisseaux et al., 2017 and Guiloski et al., 2017). This calls for the need to further investigate the influence of these aspects on DCF modes of action.

4.3. Molecular responses

Results highlighted the importance of assessing the differential responses of fish tissues in ecotoxicological studies, providing a broader view on the impacts of environmental stressors, because different tissues not only had distinct baseline levels of molecular biomarkers, but also responded differently to DCF, warming and/or acidification co-exposure. In agreement with previous findings (e.g. Islas-Flores et al., 2013; Madeira et al., 2016a, b; Maulvault et al., 2017), the present data evidenced both increase and decrease of biomarker values. Such distinct patterns were likely linked to the fact that the exposure to environmental stressors usually activates the defense and scavenging cell mechanisms (regardless of tissue) in order to overcome/adjust to the stress induced but, still, the opposite strategy (i.e. inhibition) can also occur when the stressor's severity and duration leads to the exhaustion of these biological mechanisms (Ferreira et al., 2015; Madeira et al., 2016a).

4.3.1. Antioxidant defenses

In agreement with previous findings (Islas-Flores et al., 2013; Stepanova et al., 2013; Guiloski et al., 2015), the present results showed that DCF exposure (alone) affected fish antioxidant machinery in a tissue- and biomarker-specific way, for instance, significantly inhibiting CAT and SOD activity in the muscle (but not in the liver and brain) or enhancing GST activity in the brain (but not in muscle and liver). As for the interactive effects of temperature and pCO_2 levels, previous studies have evidenced how these two stressors can strongly affect fish antioxidant machinery (e.g. Rosa et al., 2016; Maulvault et al., 2017), generally leading to increased CAT, SOD and GST activities, which matches some of the current findings (e.g. increased SOD activity in fish liver under acidification regardless of DFC exposure, as well as increased GST activity in all tissues under warming and in muscle and brain of contaminated fish under acidification), but not all. First, 5 °C and/or 1000 μatm pCO_2 increase were not sufficient to activate CAT in any tissue of non-contaminated fish, while an inhibition of this enzyme was observed in contaminated fish muscle, thus indicating that changes in CAT activity in these treatments were mostly induced by DCF, rather than by temperature and pCO_2 levels. As argued in an earlier work on *Mytilus galloprovincialis* exposed to ibuprofen (Gonzalez-Rey and Bebianno, 2011), CAT activity inhibition in DCF-exposed fish muscle could be due to an accumulation of arachidonic acid in cells due to the blockage of cyclooxygenase activity (cyclooxygenases are responsible for the conversion of arachidonic acid into prostaglandin), consequently leading to an excessive production of H_2O_2. Second, warming and acidification increased SOD inhibition in non-contaminated fish muscle (compared to contaminated samples) most likely due to ROS-mediated denaturation that inactivated this enzyme (Ferreira et al., 2015), but such effect was attenuated by DCF co-exposure. Third, acidification seemed to have compromised GST activity in muscle and liver, and this impairment could be due to a decreased ability to form reduced glutathione in this tissue, thus, resulting in reduced amount of substrate for GST to act on (Gonzalez-Rey and Bebianno, 2011).

Despite the impairment of the antioxidant machinery under the stress conditions mentioned above, fish muscle was the only tissue showing increased formation of lipid peroxides (i.e. increased MDA concentration), which occurred under warming or in co-exposure with acidification and DCF, thus suggesting that the enhancement of the antioxidant defenses only prevented oxidative stress to some extent, leading to the consequent cell damage.

4.3.2. Chaperoning and protein degradation

Corroborating some of the present findings (i.e. muscle: acidification and warming with DCF co-exposure; liver: warming with or without co-exposure with acidification; and brain: acidification and warming, with and without DCF co-exposure), the synthesis of molecular chaperones, such as heat shock proteins, can be induced in order to repair, refold, and/or eliminate damaged proteins, thus preventing the cellular damage promoted by the exposure to both abiotic stressors (e.g. Pimentel et al., 2016; Madeira et al., 2016a,b) and chemical contaminants (e.g. Maulvault et al., 2017), including NSAIDs (Gravel and Vijayan, 2007). On the other hand, as observed in fish muscle under acidification alone or in the brain of fish exposed to DCF alone or combined with the other two stressors, a down-regulation can also occur under severe and/or chronic stress conditions, due to the exhaustion of the cytoprotective systems and metabolic depression (Madeira et al., 2016a), with the lack of HSPssynthesis against proteotoxic stressors being associated with cell death (Hightower, 1991). Thus, the fact that HSP70/HSC70 synthesis was inhibited in the brain when DCF acted alone suggests that warming and acidification may have reversed the effects of DCF, but not when both stressors were combined. Previous studies have highlighted that the activation of chaperones is not a straightforward process, since the threshold for induction/repression can be influenced by several factors, including stress levels, interactions with chemical contaminants, species, tissue and animal hormone levels (e.g. Mahmood et al., 2014; Madeira et al., 2016b).

Similar to HSPs, Ub synthesis can be induced to signal that denatured proteins need to be degraded by the proteasome, but can also be inhibited under extreme or long-lasting conditions due to physiological collapse, as protein synthesis is an energy-demanding cellular process, accounting for more than 50% of fish total oxygen consumption (e.g. Gravel and Vijayan, 2007; Madeira et al., 2016a). Hence, the decrease of Ub content in fish (liver and muscle) can be explained by the lack of adequate energy for Ub formation under the co-exposure of DCF, acidification and/or warming, as opposed to non-contaminated

treatments simulating climate change effects or to DCF-exposure alone. Moreover, such impaired Ub synthesis linked with the lower cell viability and increased ENAs, further reinforces the genotoxic potential of DCF, particularly when combined with abiotic stressors.

4.3.3. Neurotoxicity and endocrine disruption

In the brain, this Acetylcholinesterase (AChE) can be a target for chemical contaminants, leading to the inhibition of its activity and, therefore, to failed synaptic transmission and muscle overstimulation (Schmidel et al., 2014; Topal et al., 2017). Furthermore, the exposure to abiotic stressors, such as increased temperature and pCO_2 levels, can alter AChE activity (Rosa et al., 2016). Contrasting to these findings, warming and acidification (alone or combined) did not affect AChE brain activity in the current study. This could be related to wide thresholds of physiological tolerance to temperature and pCO_2 changes of *D. labrax*, and/or to the longer acclimation and trial durations that could enable fish to cope with these changes. On the other hand, in agreement with the findings of Gonzalez-Rey and Bebianno (2014) for *M. galloprovencialis* and of Mathias et al. (2018) for *Rhamdia quelen*, DCF exposure alone induced AChE activity. Yet, this induction was counteracted by acidification and warming (i.e. no effect in DCF + Acid and DCF + Acid + Warm treatments, as well as a significant inhibition in DCF + Warm treatment). Moreover, the changes elicited by DCF can reflect AChE activity modulation by the antioxidant machinery and VTG-like protein levels (i.e. estrogenic activity) in fish, rather than reflecting an impaired neurotransmission function (Gonzalez-Rey and Bebianno, 2014; Oliveira et al., 2015).

Although VTG is usually undetectable in males and sexually immature individuals, its production can be induced by the exposure to estrogenic compounds (e.g. Matozzo et al., 2008; Gonzalez-Rey and Bebianno, 2014). In agreement with previous findings, VTG synthesis was induced by DCF exposure alone or combined with warming, thus evidencing the estrogenic potential of this contaminant (e.g. Gonzalez-Rey and Bebianno, 2014; Gröner et al., 2017). As for the effect of abiotic stressors, the enhanced VTG synthesis at warmer temperatures in both non-contaminated and DCF-exposed fish is consistent with previous studies involving fish species co-exposed to endocrine disrupting compounds and increased temperatures (Chandra et al., 2012; Shappell et al., 2018), and likely related to enhanced fish metabolic rates and enzymatic activities. Interestingly, acidification seemed to have had the opposite effect, inhibiting VTG synthesis when acting alone, or not significantly affecting it when co-exposed with warming and/or DCF. Previous studies with freshwater fish species pointed out that low seawater pH levels may have a significant effect on fish endocrine regulation (e.g. modulating the synthesis of cortisol and thyroid hormones which are, in turn, linked to the VTG induction; Kwong et al., 2014; McCormick and Bradshaw, 2006). Yet, little is still known regarding the interactive effects of coexposure of acidification and estrogenic contaminants, thus, calling for further investigations of the effects of environmental stressors on fish endocrine system and reproduction.

4.4. Integrating the different ecotoxicological responses

As evidenced in the present study, drawing general conclusions based on different biomarker responses is not evident since: i) some biomarkers interact with each other in complex biological mechanisms; ii) biomarkers can respond differently in each tissue according to their baseline levels; iii) an environmental stressor can elicit distinct responses when acting alone or in combination with other environmental stressor; and iv) a given stressor can activate biomarker responses to a certain extent, but also inactivate them when the degree of severity/chronicity exceeds the ability of an organism to cope with that stressor, thus either an increase or a decrease of biomarker value can indicate a lowered animal fitness (Serafim et al., 2012; Ferreira et al., 2015; Madeira et al., 2016a). Because of this, integrating the responses of different biomarkers (IBRs) can be a useful tool, highlighting patterns that are not evident when analyzing individual biomarker responses and providing a general insight of the way organisms are dealing with the exposure to environmental stressors (Serafim et al., 2012; Ferreira et al., 2015; Madeira et al., 2016a).

The fact that in the current study, the muscle (as well as the whole organisms) revealed lower mean IBR than liver and brain, is not in agreement with the trend reported by Madeira et al. (2016a), as higher IBR responsiveness was generally associated to aerobic tissues, such as muscle, gills and liver. Yet, the higher responsiveness of liver and brain observed in the present study may also be partially related to the number of biomarkers that were used to compute the IBR in each tissue (i.e. six biomarkers were used to compute the IBR in whole organism and muscle, while seven biomarkers were used for liver and brain), as the total IBR index value corresponds to the sum of all areas that connect to consecutive biomarker scores (Guerlet et al., 2010). Indeed, although previous literature does not propose an appropriate number of biomarkers to be used in IBR computations, Serafim et al. (2012), pointed out that the "relative weight" of each biomarker response is influenced by the number of biomarkers included in IBR calculations, therefore, largely affecting the final IBR value, and with a higher number of biomarkers generally yielding higher IBR indexes.

Regardless of the number/type of analyzed biomarkers, overall, higher IBR values have been associated to the exposure to both chemical contaminants (Serafim et al., 2012; Ferreira et al., 2015; Ács et al., 2016) and increased temperatures (Kamel et al., 2014; Madeira et al., 2016a), whereas no studies with biomarkers' IBR linked to acidification were found. Indeed, DCF dietary exposure was, in overall, associated to higher IBR values for the whole organism, muscle and brain, indicating a decreased fish health condition, thus, confirming the toxicological attributes of this contaminant. On the other hand, warming alone resulted in higher IBR value in the liver, and that is likely related to increased metabolism and intense enzymatic activity induced by increased temperatures in this organ.

Results pointed out that the selected biomarkers for the whole organism (i.e. K, HSI, BB_{ratio} and hematological parameters) were more responsive to the co-exposure of DCF and abiotic stressors associated with climate change. In contrast, less consistent tissue-specific patterns were found in muscle, liver and brain, especially when DCF co-exposure occurred, owing to the high variability of molecular responses observed (i.e.up- or down-regulations). The overall higher IBR values registered in treatments simulating warming alone (in liver and brain) or in co-exposure with DCF (in liver and muscle) evidenced the inability of fish to compensate for the stress induced by a 5 °C increase. On the other hand, the role of acidification in lowering fish fitness/physiological condition was not so evident. The lowered IBR values found in fish brain exposed to DCF + Acid and DCF + Warm treatments suggested that, despite the elicited biomarker changes, fish were able to better cope with stressors than those exposed to DCF, acidification and warming acting alone or to the three stressors simultaneously, likely due to the activation of antioxidant/detoxifying (GST) and chaperoning (HSP70/HSC70) mechanisms. Although the effects of warming on fish muscle, at a first glance, may seem to have been reversed by the co-exposure to acidification and DCF (i.e. lower IBR index value of DCF + Acid + Warm treatment), the IBR reduction can be explained by the inhibition of CAT activity and Ub synthesis in relation to the Control treatment and, therefore, may not be necessarily indicative of a better health condition of fish. The same argument can also be applied to the lowered liver IBR under acidification (i.e. in Acid and DCF + Acid treatments), as the first treatment triggered the inhibition of SOD and GST activities, as well as VTG content, whereas the second treatment inactivated SOD activity and Ub synthesis. Such findings highlight that, despite providing a broader overview of fish physiological condition, the use of IBRs has also limitations, as it provides a qualitative (and not quantitative) evaluation of the degree of stress induced in fish, therefore, requiring critical interpretation and background knowledge

(Serafim et al., 2012).

5. Conclusions

This study provided novel data and a contribution to fill a major research gap of the present: the interactive effects between climate change and chemical contaminants on the ecotoxicological responses of marine fish species. Results confirmed that the juvenile marine fish responses (i.e. animal fitness, immunity, cellular defense and scavenging mechanisms) to dietary DCF exposure are strongly affected by increased temperature (+5 °C) and pCO_2 (+1000 μatm). Such alterations can correspond to either the enhancement of biomarker responses (e.g. erythrocyte viability was further reduced, as well as brain GST activity and muscle Ub synthesis further increased by the combination of DCF and acidification and/or warming) or to an inhibition (e.g. lower CAT and SOD inhibition when DCF exposure was combined with acidification and/or warming, as well as lower VTG synthesis when DCF exposure was combined with warming, compared to DCF acting alone). Based on the integrated approach used in this study (i.e. the IBRs), which combined the different biomarker responses in an organism or tissue overall, DCF exposure induced more severe stress responses when fish were co-exposed with warming, compared to DCF acting alone or combined with acidification. Although IBRs proved to be a helpful tool to better understand the severity of the three studied stressors (revealing trends that were not so clear when analyzing biomarker responses individually), the limitations of their use were also evidenced in this study.

Finally, to sum up, the distinct effects promoted by DCF exposure, acidification and warming acting alone or combined highlighted the need to consider interactions between environmental stressors in future ecotoxicological studies. Such knowledge will provide wider insights on the toxicological impacts of climate change, as environmental stressors will unlikely occur in isolation, nor will the alterations of environmental conditions affect marine ecosystems in the same way across the planet.

Ethical statement

Fish trials were conducted according to legal regulations (EU Directive 2010/63), and approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University, overseen by the Portuguese National Competence Authority (Direção-Geral de Alimentação e Veterinária, DGAV). All researchers and technicians involved in the maintenance, handling and sampling of live animals were certified in Laboratory Animal Sciences, by the Federation of European Laboratory Animal Science Associations (FELASA).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2018.06.016.

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